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Intracellular signalling pathways associated with the glucose-lowering effect of ST36 electroacupuncture in streptozotocin-induced diabetic rats

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ABSTRACT

Background and aim Previous animal studies have reported a glucose-lowering effect of electroacupuncture (EA) and suggested that the mechanisms are closely related to intracellular signalling pathways. The aim of this study was to screen for potential intracellular signalling pathways that are upregulated by EA at ST36 bilaterally in rats with diabetes mellitus (DM) using microarray analysis.

Methods Streptozotocin (STZ)-induced diabetic rats were randomly assigned to experimental (EA, n=8) or control (non-EA, n=8) groups. Plasma glucose levels were measured at baseline and after 30 and 60 min, and microarray analysis was performed on samples of gastrocnemius muscle.

Results Relative to baseline values, EA significantly reduced plasma levels of glucose at 30 and 60 min. The microarray pathway analysis showed that cell adhesion molecules and type 1 DM gene sets were both upregulated in EA versus non-EA groups ($p<0.05$).

Conclusions Cell adhesion molecules might be related to the glucose-lowering effect induced by EA in rats with STZ-induced type 1 diabetes. Further research will be required to examine the involvement of related intracellular signalling pathways.

INTRODUCTION

In recent years there has been a growing interest in the glucose-lowering effects of EA. We previously found that a specific EA frequency applied bilaterally at ST36 *Zusanli* induced a significant reduction in plasma glucose. The mechanism by which plasma glucose levels are lowered is believed to involve the promotion of β -endorphin secretion, which stimulates the secretion of insulin.¹ In an adrenalectomy animal model, we found that 15 Hz

EA stimulation at CV12 *Zhongwan* also exerted a plasma glucose-lowering effect.² However, EA at ST36 lowered glucose levels to a greater degree than EA at CV12.³ In the streptozotocin (STZ)-induced rat model of type 1 diabetes mellitus (DM), the application of 15 Hz EA at ST36 induced a glucose-lowering effect that could be blocked by atropine. The mechanism of action behind the glucose-lowering effect of EA has been shown to involve modulation of the cholinergic nerves.⁴ Zhao⁵ proposed that EA triggers an immune response due to release of acetylcholine by surrounding cholinergic nerves. The STZ-induced diabetic rat is a good model to explore the non-insulin dependent hypoglycaemic pathway.

Investigations into the pathogenesis and mechanism of DM have demonstrated the involvement of signalling pathways related to the metabolism of glucose, such as those responsible for the uptake and metabolism of glucose in specific organs. Many studies have investigated the roles of the insulin receptor (IR) and glucose transporters (GLUT) in diabetes. Recently, research efforts have been directed towards investigating peroxisome proliferator-activated receptors (PPAR) for the treatment of DM.⁶ Therefore, the main pathways involved in the action of IR and GLUT, as well as the expression of PPAR-related genes, have become the subject of considerable research interest. When 3T3-L1 cells are stimulated by insulin, they modulate the amount of GLUT in the cell membranes via two different pathways to promote the intake of glucose into the intracellular space. The first pathway occurs via

activation of the IR- β subunit by insulin, followed by phosphorylation of the IR substrate. This event activates phosphatidylinositol 3-kinase (PI3K), leading to activation of downstream protein kinase C and protein kinase B (Akt) to promote the transportation of GLUT and glycogenesis.⁷ The second pathway occurs via the activation of Ras by insulin stimulation⁸ and the activation of transcription factors (such as PPAR and AP-1) of the mitogen-activated protein kinase (MAPK)-related pathways, which lowers blood sugar by increasing the production of GLUT-4. Following induction by insulin, GLUT-4 is transported from the intracellular space to the plasma membrane.⁹ GLUT plays an important role in the regulation of the insulin signalling pathway and is regarded as an important biomarker in DM.

Data on expression of all genes in a biological sample can be achieved in one experiment using the microarray method, the results of which can be analysed to determine the potential pathways involved in a given process and identify potential therapeutic targets. In a study by Gao *et al*,¹⁰ microarray analysis was used to identify differences between individual rats in the analgesic effect of ST36 EA at 1 Hz frequency. Chae *et al*¹¹ also used cDNA microarrays to investigate acupuncture analgesia. However, to date, few studies have used microarrays to investigate the molecular mechanisms behind the glucose-lowering effect of EA. Furthermore, the potential cellular mechanisms underlying activation of the glucose transport system in response to EA have not yet been studied in animal skeletal muscle preparations. Therefore, the purpose of the present study was to examine (using microarray analysis) the effect of EA on relevant intracellular signalling pathways in muscle using the STZ-induced rat model of insulin dependent DM (IDDM).

METHODS

Animal model

Normal male Wistar rats weighing 250–350 g and aged 8–10 weeks were purchased from the BioLASCO animal centre. Type 1 IDDM was induced by administration of STZ (60 mg/kg intravenously) via the femoral vein in the morning after a fasting period, as described previously.¹² The animals were housed in Plexiglas cages at a constant room temperature of $22 \pm 2^\circ\text{C}$ with a relative humidity of $65 \pm 5\%$. The rats were fed standard rat chow and given free access to water. Experiments began after an adaptation period of 1 week.

Electroacupuncture

Acupuncture points were located based on the measurement of body length as described previously.¹³ The ST36 acupuncture point was located on the anterior tibia muscle at approximately the upper one-sixth of the length of the lower leg below the knee. ST36 was

needled bilaterally perpendicularly to a depth of 2 mm with a 1.27 cm 32 gauge acupuncture needle (U Kwong Co Ltd, Taipei, Taiwan). After a 5-min needling period, EA was performed for 60 min at a frequency of 15 Hz and an amplitude of 10 mA using a HANS LY257 acupuncture point and nerve stimulator (Healthtronics, Singapore).

Experimental protocol

To assess the glucose-lowering effect of EA, STZ-induced diabetic rats (N=16) were randomly divided into an EA group (N=8) and a control group (N=8). Animals in the EA group were anaesthetised using pentobarbital (40 mg/kg intraperitoneally), then subjected to EA for 60 min. Rats in the control group were anaesthetised in the same manner but were not treated with EA. Approximately 0.3–0.5 mL of blood was collected from the femoral vein (using a heparinised 1 mL syringe) for glucose testing immediately prior to the experiment and after 30 and 60 min intervals, as previously described. Blood was transferred into Eppendorf tubes, lightly shaken and stored on ice. Following centrifugation at $21\,880 \times g$ for 5 min, a glucose UV reagent (Raichem, USA) was added and the serum glucose level was determined using a fully automated biochemical analyzer (Roche COBAS-MIRA-PLUS, USA). At the end of treatment (60 min), portions of the gastrocnemius muscles were collected and snap frozen.

RNA extraction

Total RNA was extracted from skeletal muscle using an RNeasy Mini kit (Qiagen, Valencia, California, USA) and quantified using the Beckman DU800 spectrophotometer (Beckman Coulter, Fullerton, California, USA). Samples with A260/A280 ratios >1.8 were further evaluated using an Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, California, USA). RNA samples with an RNA integrity number >7.0 were accepted for subsequent microarray analysis.

Microarray

Microarray analysis was performed as previously described.^{14, 15} Briefly, after purification, 750 ng of cDNA was hybridised to the Illumina Sentrix Rat Ref-12 BeadChip microarray (Illumina, San Diego, California, USA) representing approximately 22 000 genes. The procedure followed the standard protocol for Illumina Whole Genome Gene Expression with IntelliHyb, revision A. Hybridisation was detected using 1 $\mu\text{g/mL}$ Cy3-streptavidin (GE Healthcare, Little Chalfont, UK). The array was scanned with a BeadArray Reader (Illumina). Expression values were generated with the Bead Studio V2.3.1 software (Illumina). The detection of significant genes followed the standard protocol, including normalisation and adjustment for multiple comparisons. We also

Figure 1 Glucose-lowering effect of electroacupuncture (EA). Serial measurements of plasma glucose at baseline (following a 12 h fast) and 30–60 min later in rats given streptozotocin to induce diabetes mellitus followed by electroacupuncture (EA) at ST36 (EA group, $n=8$) or no treatment (non-EA group, $n=8$). ** $p<0.01$, Student t test.

undertook Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis to investigate whether EA impacted on the expression of specific gene sets.

Statistical analysis

All values are expressed as mean $\pm$ SEM. The area under the curve (AUC) was calculated for comparisons using the statistical software MedCalc. Differences in mean values between the two groups were determined using a Student unpaired t test with the p value significance set as $p<0.05$. A paired t test was used to determine significant changes in the levels of plasma indicators before and after treatment. For all comparisons, a two-sided p value <0.05 was considered to represent statistical significance. For the results of the microarray, the Benjamin Hochberg false discovery rate (BH-FDR) was used to control for multiple comparisons and was set at a threshold of 5% (<0.05). This rate was applied for the exploration of differential gene expression between the EA-treated and control groups.

RESULTS

Glucose-lowering effects of EA

Figure 1 illustrates the changes in blood glucose over time in the EA and control (non-EA) groups. After 60 min the mean plasma glucose level in the EA group was lower than in the control group (178.5 ± 58.4 mg/dL vs 369.6 ± 23.3 mg/dL, $p<0.05$). The AUC for glucose was also reduced in the EA group compared with the non-EA group ($14\,769 \pm 3239$ vs $22\,179 \pm 1691$ mg/dL/min, $p<0.05$).

Pathway analysis

Microarray analysis of gene expression in muscle tissue revealed that two pathways—specifically, type 1 DM and cell adhesion molecules—were significantly upregulated following EA treatment ($p<0.05$, $FDR<0.05$, table 1). Although several other gene sets also appeared to be upregulated by EA, these did not meet our predetermined threshold for false discovery. There were no statistically significant differences between EA and non-EA treated groups in leucocyte transendothelial migration, cytokine–cytokine receptor interactions or in the MAPK, PPAR, Wnt, insulin or chemokine signalling pathways.

DISCUSSION

In this study, 15 Hz EA at ST36 in STZ-induced diabetic rats reduced blood sugar levels, as demonstrated by the significant differences in serum glucose between the EA and non-EA groups at the 30 and 60 min time points. We also used a microarray to examine various signalling pathways that may contribute to the glucose-lowering effect of EA and to screen for relationships between these gene sets and DM. This analysis revealed that cell adhesion molecules and type 1 DM pathways were significantly associated with the glucose-lowering effect. The other seven pathways examined (leucocyte transendothelial migration, cytokine–cytokine receptor interactions, and MAPK, PPAR, Wnt and insulin signalling pathways),

Table 1 Pathway analysis

Pathway	Total no of genes	No of genes upregulated	No of genes downregulated	p Value
Type I diabetes mellitus	63	5	0	0.021
Cell adhesion molecules	147	6	2	0.023
MAPK signalling pathway	254	6	4	0.126
Leucocyte transendothelial migration	117	4	1	0.142
PPAR signalling pathway	79	1	2	0.254
Wnt signalling pathway	106	2	3	0.261
Cytokine–cytokine receptor interaction	241	4	1	0.479
Insulin signalling pathway	91	3	1	0.368
Chemokine signalling pathway	7	4	1	0.386

Total relative gene expression of various pathways including number of genes found to be upregulated (fold change >1.5) and downregulated (fold change <1.5) in the electroacupuncture (EA) group compared with the control (non-EA) group ($n=8$ each).

MAPK, mitogen-activated protein kinase; PPAR, proliferator-activated receptors.

although regulated in muscle tissue, were not significantly affected by EA treatment in the present study.

Cell adhesion molecules, leucocyte transendothelial migration, cytokine–cytokine receptor interactions and the chemokine signalling pathway are related to immune and inflammatory responses.^{16–19} The significant change in cell adhesion molecules may represent an underlying mechanism of action for the effects of EA in STZ-induced diabetic rats, but we cannot rule out a more direct inflammatory effect on muscle cells triggered by mechanical stimuli via EA.

There are some limitations to this study. For example, we were unable to confirm the definitive signalling pathway involved in the glucose-lowering effect of EA in STZ-induced diabetic rats. Additionally, we did not verify the microarray results or carry out any functional research into the selected pathways. Instead, this experiment was intended to serve as a screening study. Analysis of specific pathways using functional studies will be investigated in the future.

Although no effects were seen on most pathways, many of those examined in the present study have been implicated physiologically in DM and glucose metabolism. For example, the p38 MAPK subfamily has been proposed to play a role in activating the insulin-dependent glucose transport system.^{16–19} A number of studies support the potential direct or indirect role of p38 MAPK in the regulation of the glucose transport system in insulin-sensitive tissues including skeletal muscle,^{16 17 19 20} although this concept remains controversial.^{21–23} Moreover, the Wnt/ β -catenin system is a glucose-responsive signalling system that plays a role in pathways involved in sensing changes in metabolic status.²⁴ The existence of crosstalk between these pathways and Wnt/ β -catenin signalling in skeletal muscle suggests that organ-selective modulation of Wnt signalling might be possible. Wnt signalling may also be involved in the regulation of glucose homeostasis, and Wnt/ β -catenin signalling may induce GLUT-4 translocation to the plasma membrane through an insulin-independent pathway.²⁵

The pathway analysis based on our microarray data showed that cell adhesion molecules might be involved in the glucose-lowering effect of EA in rats with STZ-induced DM. However, it remains possible that different signalling pathways might be involved in different animal models of diabetes and, indeed, the human condition. Further studies are needed in the future to elucidate these effects.

CONCLUSION

Cell adhesion molecule and type 1 DM signalling pathways appear to be involved in the glucose-lowering effect of EA at ST36 in rats with STZ-induced diabetes. However, further research is

required to determine the precise underlying mechanisms of action.

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Competing interests None declared.

Ethics approval The protocol for this study was approved by the ethical review committee of China Medical University, Taichung, Taiwan. All procedures were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

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